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Structural insights into hepatitis C virus neutralization

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Inspite of the available antiviral therapy, hepatitis C virus (HCV) remains a global health burden and a prophylactic vaccine would help to eliminate the risk to develop chronic liver diseases. Structural insights into the function of the glycoproteins E1 and E2 in virus entry and the interplay with the host's humoral immune response are key for informed vaccine development. We review recently reported structural insights into receptor binding of HCV glycoproteins and the assembly of an intact membrane-bound E1–E2 heterodimer. These data are used together with available functional data to draw a simplified model of virus entry, which highlights gaps in our current knowledge that warrant further research to fully understand this process at the atomic level.

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Introduction

Approximately 58 million people worldwide are estimated to be chronically infected with the hepatitis C virus (HCV) [1]. About 70% of HCV infections progress to the chronic phase, which can lead to severe liver damage such as liver cirrhosis and hepatocellular carcinoma (HCC). Direct-acting antiviral agents (DAAs) are available and have an impressive high cure rate of more than 95% [2]. However, the acute phase of HCV infection is mostly asymptomatic, resulting in a low awareness of the infection and subsequently a late diagnosis and treatment [3]. After viral clearance, function and metabolism of HCV-specific CD8+ T cells remain impaired [4], and patients cured by DAAs, especially in high-risk groups such as intravenous drug users, often get reinfected [5]. Moreover, cured individuals may still develop progressive liver failure and HCC [6,7], illustrating the urgent clinical need for a prophylactic HCV vaccine.

Accumulating evidence suggests that both B- and T-cell immunity are important for the control of an acute HCV infection (reviewed in [8,9]). To date, two prophylactic vaccine candidates have progressed to clinical trials in humans, but with limited success. An adenoviral vector-based T-cell vaccine candidate encoding the non-structural proteins of a single HCV isolate failed to protect against chronic HCV infection in a cohort of people who inject drugs at high risk of virus transmission [10]. Even though a B-cell vaccine candidate comprising MF-59-adjuvanted viral glycoprotein E1/E2 heterodimers of a single HCV isolate protected chimpanzees from challenge with HCV of the same genotype, in a phase-I trial, broadly neutralizing antibodies (bnAbs) were only induced in a small fraction of individuals [11,12]. Hence, an effective prophylactic HCV vaccine likely requires an immunogen that robustly elicits bnAbs in combination with an efficient T-cell response [13].

BnAbs protect against HCV infection in humanized mice [14] and chimpanzees [15] and HCV viral clearance is associated with a robust induction of bnAbs at an early time point of the infection [16]. The major neutralization epitopes are located on the two HCV glycoproteins E1 and E2. These two form heterodimers and higher oligomers such as trimers of the heterodimer within the viral envelope [17]. During virus entry, the E1–E2 glycoprotein complex interacts with the scavenger receptor class-B member 1 (SR-B1) [18] and the cluster of differentiation 81 (CD81) [19,20], thereby activating signaling pathways that promote HCV–CD81 movement to tight-junction regions, where interactions with claudin 1 and occludin support viral internalization [21,22].

The larger glycoprotein E2 comprises at least three regions with high genetic diversity termed hypervariable regions (HVRs) 1, 2 and intergenotypic variable region. Neutralizing antibodies (NAbs) targeting HVR1 are mostly strain-specific and resistance develops rapidly [9,23]. Moreover, due to the conformational flexibility in this region, anti-HVR1 Abs seem to hinder binding of other bnAbs in a sterical manner [24] and the removal of HVR1 leads to an increased sensitivity to nAbs [25,26]. Simultaneously, E2 also comprises the conserved

discontinuous CD81-binding site and conserved neutralization epitopes, which have high barrier to resistance and are critical to guide vaccine development [27]. These bnAb epitopes overlap mainly with the CD81-binding site. In addition, bnAbstargeting epitopes on the smaller glycoprotein E1 [28] and the E1–E2 heterodimer outside the conserved CD81-binding site have been reported [29].

While virus attachment to cellular receptors SR-B1 and CD81 is mediated by the glycoprotein E2, the precise role of E1 as well as the chronological order of events during later steps of virus entry such as the membrane fusion process remains poorly understood to date. It even remains unclear, which of the two glycoproteins serves as viral fusion protein, that is, facilitates the fusion of the viral envelope with a membrane of the target cell. A putative fusion peptide within E1 has been proposed [30–32], however, its precise role in membrane insertion and merging of two double-layer membranes has not yet been elucidated.

Structural flexibility of soluble E2 ectodomains and neutralization epitopes

Structural data are critical for informed B-cell vaccine design, and in the last decade, soluble E2 ectodomain (sE2) core fragments have been structurally and antigenically characterized in complex with several fragments from human [33–39], murine [40], and macaque [41] monoclonal antibodies (mAbs). The E2 ectodomain core consists of a central Ig-fold β-sandwich flanked by two additional protein layers — the back layer and the front layer [33] containing the composite CD81-binding site — and is stabilized by numerous disulfide bonds [33,34]. Combined peptide and alanine scanning together with mAb cross-competition studies have yielded different, but partially overlapping nomenclature systems to describe and cluster neutralization and non-neutralization, linear and conformational epitopes on E2 such as antigenic domain A–E, antigenic region (AR) 1–3, or epitope I/II, including the adjacent loop named CD81-binding loop (epitope III) [42,43]. More recently, the two conformational antigenic sites (AS) 108 and 146 on E2 and E1–E2, respectively, were described using alanine scanning and mAb competition experiments [44]. Both AS are distinct from previously defined AR1–5 [14], but a closer relationship of AS108 and AS146 to domain A–E cannot be excluded [44]. Using available structural information, ARs have been mapped onto the 3D structure of the E2 ectodomain core, revealing in several cases (such as the bnAb AR3C [33]) important differences in the contact residues identified in structural and functional analyses. This highlights that the gold standard to identify neutralization epitopes remains the structural analysis of the immune complex.

Structural studies on important neutralization epitopes revealed an extensive conformational flexibility in epitope I (aa412–423) [24,34,45–51] and the highly conserved E2 front-layer epitope II [16,33–37,39], which also extends to the Ig-like domain within E2 [20,33,40,52]. This observation led to the hypothesis that different conformational states such as a 'closed' and an 'open' conformational state of E2 (or the E1–E2 heterodimer) may be associated with different functional states during virus entry, such as coreceptor binding and antibody neutralization [36]. Hydrogen–deuterium-exchange (HDX) mass spectrometry studies confirm that in particular epitope I, the E2 front layer and the CD81-binding loop are remarkably flexible [40,49]. The high global HDX rate observed for E2 is comparable to the one of HIV-1 gp120 [49], another viral glycoprotein with remarkable structural flexibility that undergoes a range of conformational changes during entry (reviewed in [53]).

Many viral glycoproteins undergo conformational changes during virus entry, ultimately leading to an energy release that helps overcoming the large energy barrier to merge two lipid bilayer membranes (reviewed in [54]). A number of virions, including, for example, flaviviruses or picornaviruses, have been described to be dynamic structures that exist as an ensemble of states with distinct important biological roles, characterized by reversible changes in glycoprotein structure or arrangement, referred to as virus breathing [55]. A similar dynamic behavior has also been observed for HCV in time- and temperature-dependent neutralization experiments [56], but has not yet been structurally characterized in detail.

CD81-receptor binding and membrane insertion

More insights into the functional relevance of the conformational flexibility within HCV E2 were recently provided by the crystal structure of sE2 in complex with the large extracellular loop of tamarin CD81 (tCD81 LEL) [20]. Two major conformational changes in sE2 occur upon CD81 binding. First, residues 418–424, against which a retracted conformation of the CD81-binding loop partially packs in the absence of CD81 binding [33–35,37], appear to be stabilized by CD81 binding in an alternative conformation. This alternative conformation of residues 418–424 allows for an extension of the CD81-binding loop by 13–15 Å so that it can pack partly against the tCD81 LEL [20].

The structural characterization of the discontinuous CD81-binding site on HCV E2 allows the analysis of receptor-binding determinants, including residues within epitopes I and II, the CD81-binding loop, and the back layer. Surprisingly, the strictly conserved E2

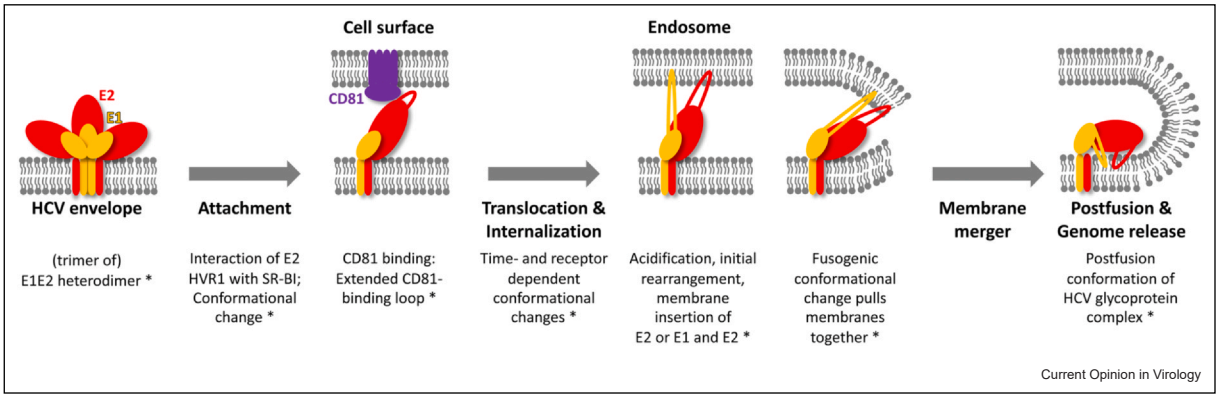
residue W420 within epitope I previously identified as an essential CD81-binding residue [57] and a contact residue for several bnAbs [45,46] does not directly interact with CD81. Its sidechain rather packs against a hydrophobic region on the inner sheet of the E2 Ig-like core to constitute a part of the CD81-binding interface and allow wrapping of residues 416–422 around CD81, hence playing a rather indirect role for CD81 binding. Similarly, G532 and D537 (or G530 and D537 according to H77 polyprotein numbering), previously identified using alanine scanning mutagenesis to be key for the interaction with immobilized CD81 [58], are not involved in a direct interaction with CD81 but likely favor the extended loop conformation through interactions with residue 424.

Similar to many other enveloped viruses, HCV entry depends on endosomal acidification, but rate-limiting, post-binding events appear to be required to render HCV particles competent for acid-triggered membrane fusion [59]. Indeed, membrane insertion of sE2 is primed by both acidic pH and CD81 binding using liposome flotation experiments [60]. Of note, the structure of sE2 in complex with the tCD81 LEL reveals that two large aromatic residues Y529 and W531 in the tip of the CD81-binding loop extend toward the membrane bilayer (Figure 1). In such an environment — with a flexible membrane bilayer and hydrophilic phospholipid head groups extending around 3–5 Å away from the hydrophobic core — insertion of Y529 and W531 sidechains into the outer leaflet of the membrane would be energetically favorable [20]. Interestingly, the amino acid at position 530 is not conserved, but generally polar, which could help to orient Y529 and W531 relative to the lipid head groups. The crucial role of these two aromatic residues for membrane insertion was confirmed by mutagenesis in pH-dependent liposome flotation experiments [20].

In addition, a conserved region within E1 containing a putative internal fusion loop (IFL) (residues 272–286) may also contribute to insertion of HCV glycoproteins into the endosomal membrane [61,62]. Indeed, an extensive mutagenesis study on both HCV glycoproteins demonstrated five residues within this loop (S273, Y276, G282, F285, and L286) to be critical for infectivity without affecting E1 and E2 folding, E1–E2 assembly, CD81-LEL binding, and incorporation into HCVpp [63]. The authors proposed E1 to undergo low-pH-induced conformational changes to form an extended intermediate conformation that facilitates membrane insertion of the IFL.

In general, protonation of histidine acid sidechains due to endosomal acidification often causes conformational changes within viral fusion proteins required to induce membrane fusion [64]. Based on alanine scanning mutagenesis, E2 H691, E2 H693, and E1 H261 were identified to be key players for the rearrangement of the HCV glycoprotein complex before membrane insertion [63]. In HCV E2, two histidine residues, H421 and H445, are in close proximity to CD81 but distant from the extended CD81-binding loop. Enhanced viral entry and cell–cell fusion at neutral and low-pH values were reported for a H445R mutant, suggesting that the protonation state of H445 could act as a regulator of HCV fusion [65]. In analogy to other viral fusion proteins [66], it is assumed that a low-pH-induced fusogenic conformational change subsequently brings the viral transmembrane helices of E2 (residues 718–746) and E1 (residues 350–382) into close proximity to the site of membrane insertion (i.e., the CD81-binding loop and

Figure 1



A simplified model of the HCV entry pathway focused on the role of the glycoproteins. Recent functional and structural data were used to draw a model to illustrate the current understanding how HCV glycoproteins contribute to HCV entry. While on the left the proposed trimer of the E1–E2 heterodimer is shown, in subsequent steps, only one E1–E2 heterodimer is shown for clarity. Gaps in our current understanding are highlighted by asterisks.

the IFL within E1) for subsequent membrane fusion. The base and stem region of E2 (residues 645–700 and 701–717) and the E1 stem (residues 315–346) could possibly contribute to bridging these distances of about 40–60 Å [20,67].

Structure of an E1–E2 heterodimer

Intrinsic flexibility, conformational heterogeneity, disulfide bond scrambling, and abundant glycosylation has hampered structure determination of a full-length E1–E2 heterodimer for many years (reviewed in [68,69]). Alanine scanning mutagenesis provided additional insights into this glycoprotein assembly, revealing that E1–E2 heterodimer is a fragile protein complex since single-alanine mutations at 92% of all residue positions compromise its function [63].

Recently, the cryo-EM structure of a membrane-extracted full-length E1–E2 heterodimer reconstituted into peptide discs and in complex with bnAbs shed light onto the predominantly hydrophobic interactions between the two glycoproteins at the atomic level [67]. Using the obtained map, 51% of E1 and 82% of E2 were built, leaving out potentially flexible regions, including the HVR1, the N-terminal part of epitope I of E2, the putative fusion peptide of E1, and both transmembrane domains (TMDs). A short helix within the E1 stem targeted by neutralizing antibodies (residues 314–324, [28]) aligns well with its previously reported structure in complex with a nAb fragment [70], but the observed conformation of the N-terminal domain of E1 in the cryo-EM structure differs substantially from the crystal structure of an N-terminal E1 fragment [67,71], confirming that E2 is required to be present for correct folding of E1. The C-terminal base of E2 (residues 644–700) consists of an extended loop and the stem region (residues 701–717) connecting the base with the TMD. The interaction between E1 and E2 is non-covalent and features a deep hydrophobic cavity in the base of E2 packing against E1, a hydrogen bonding network and two highly conserved glycans in E1, which contribute to the E1–E2 interaction, in line with previous glycosylation mutagenesis studies [72].

The visualized dense glycan shield on E1–E2 is mostly located on the neutralizing face of the E1–E2 glycoprotein complex, whereas the opposite face is rather hydrophobic and highly conserved — suggesting that this non-neutralizing face is buried in infectious particles [67], possibly in an oligomeric assembly of the HCV glycoprotein heterodimer [17] or by direct contact with lipoproteins. The TMDs of both glycoproteins remained unresolved in the cryo- EM structure, but AI-based structure prediction in combination with the experimentally derived model allowed the positioning of the E1–E2 heterodimer relative to the membrane [67]. Structural superposition of this full-length E1–E2 heterodimer within its membrane environment onto the tCD81–sE2 complex [20] and full-length human CD81 (PDB: 5TCX [73]) suggests that for binding of full-length CD81 in the target membrane, an additional rearrangement of the HCV glycoprotein complex might be required. Of note, a range of tilt angles from the membrane in all radial directions has been previously shown for other type-I membrane proteins (e.g. the epithelial fusion failure 1, [74]). It is tempting to speculate whether the E1–E2 heterodimer could also display such a tilting flexibility and thereby protrude from the membrane to allow for CD81-receptor binding. Alternatively, additional rearrangements within the glycoprotein complex could allow binding to CD81 within the plasma membrane. Such a rearrangement would also facilitate the recognition by non-neutralizing mAbs targeting epitopes that face the membrane in the current model [34,36,67]. Hence, further structural studies in the membrane context (with a particular focus on cryo electron tomography) and in particular in the context of an assembled oligomeric glycoprotein complex are required to understand the individual conformational changes of the two glycoproteins occurring during virus entry in full detail.

Conclusion

The recent progress described above paves the way to draw a simplified model of HCV entry, including both receptor binding and membrane fusion, which highlights where essential information is still missing to fully understand HCV entry at the atomic level (Figure 1, asterisks). A key question remains the precise arrangement of HCV E1–E2 heterodimers at the virion surface (possibly as trimers of heterodimers [17]). HCV initially attaches to surface proteoglycans, lipid receptors such as SR-B1 (reviewed also in [75]) and CD81, and it remains poorly understood to date, how SR-B1 binding impacts on glycoprotein structure and subsequent steps of the complex entry process. CD81 binding brings the tip of the CD81-binding loop of E2 toward the membrane bilayer [20], likely constituting the first step toward subsequent membrane insertion. Next, HCV virions are translocated in complex with CD81 toward tight junctions and time- and receptor-dependent conformational changes, which yet remain to be characterized, possibly take place [75]. At the tight junctions, the 'late' entry factors Claudin-1 and Occludin support productive uptake of the virus into cells and HCV virions are taken up into endocytic vesicles [75]. Subsequently, endosomal acidification occurs and likely induces a low-pH rearrangement within E1 (and possibly an additional one in E2). With the help of the increased electrostatic potential of E2, this rearrangement results in membrane insertion of the E2 CD81-binding loop tip [20] and possibly in parallel of the E1 IFL [63]. Subsequently, a

fusogenic conformational change is required to induce merging of the viral envelope with the endosomal membrane. This has been proposed to involve packing of the E1 stem region to the side of the IFL, resulting in a hairpin conformation that pulls the two bilayer membranes together [63].

In particular, the latter steps of this model remain poorly characterized to date, and further research is needed to understand the individual structural rearrangements within the HCV glycoprotein complex at the atomic level.

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Data Availability

No data were used for the research described in the article.

Conflict of interest statement

The authors declare no conflict of interest.

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Owing to strict space limitations, we could only cite and discuss a very limited number of the important findings in the field. Hence, we apologize to all our colleagues who have contributed to this area of research but whose work could not be discussed.

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- •• of outstanding interest

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